

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**  
**REQUEST FOR FILING NATIONAL PHASE OF**  
**PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495**

09/806564

To: Hon. Commissioner of Patents  
 Washington, D.C. 20231

TRANSMITTAL LETTER TO THE UNITED STATES  
 DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: P 0279469 /202382/CMH/dg  
M# /Client Ref.

From: Pillsbury Winthrop LLP, IP Group:

Date: March 30, 2001

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

- |   |   |   |
|---|---|---|
| 1. International Application<br><br><u>PCT/GB99/03258</u><br>↑ country code | 2. International Filing Date<br><br><u>1 October 1999</u><br>Day MONTH Year | 3. Earliest Priority Date Claimed<br><br><u>1 October 1998</u><br>Day MONTH Year<br>(use item 2 if no earlier priority) |
|---|---|---|
4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☐ 20 months from above item 3 date      (b) ☒ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is April 1, 2001

5. Title of Invention CHARACTERISING POLYPEPTIDES THROUGH CLEAVAGE AND MASS SPECTROMETRY

6. Inventor(s) Gunter Schmidt and Andrew Hugin Thompson

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

7. ☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).
8. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:
- a. ☒ Request;
- b. ☒ Abstract;
- c. 28 pgs. Spec. and Claims;
- d.        sheet(s) Drawing which are ☐ informal ☐ formal of size ☐ A4 ☐ 11"
9. ☒ A copy of the International Application has been transmitted by the International Bureau.
10. A translation of the International Application into English (35 U.S.C. 371(c)(2))
- a. ☐ is transmitted herewith including: (1) ☐ Request; (2) ☐ Abstract;
- (3)        pgs. Spec. and Claims;
- (4)        sheet(s) Drawing which are: ☐ informal ☐ formal of size ☐ A4 ☐ 11"
- b. ☒ is not required, as the application was filed in English.
- c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
- d. ☐ Translation verification attached (not required now).
11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:
- a. ☒ --This application is the national phase of international application PCT/GB99/03258 filed October 1, 1999 which designated the U.S. and that international application ☒ was ☐ was not published under PCT Article 21(2) in English.--
- b. ☐ --This application also claims the benefit of U.S. Provisional Application No. 60/      , filed       .--

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12. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., before 18th month from first priority date above in item 3, are transmitted herewith (file only if in English) including:
13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau
14. ☐ Translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of claim amendments made before 18th month, is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).
15. **A declaration of the inventor (35 U.S.C. 371(c)(4))**  
 a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy  
 b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
16. **An International Search Report (ISR):**  
 a. Was prepared by ☒ European Patent Office ☐ Japanese Patent Office ☐ Other  
 b. ☒ has been transmitted by the international Bureau to PTO.  
 c. ☒ copy herewith (2 pg(s).) ☒ plus Annex of family members (1 pg(s).).
17. **International Preliminary Examination Report (IPER):**  
 a. ☒ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.  
 b. ☒ copy herewith in English.  
 c.1 ☐ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:  
 c.2 ☐ Specification/claim pages # \_\_\_\_\_ claims # \_\_\_\_\_  
 Dwg Sheets # \_\_\_\_\_  
 d. ☐ Translation of Annex(es) to IPER (required by 30<sup>th</sup> month due date, or else annexed amendments will be considered canceled).
18. **Information Disclosure Statement including:**  
 a. ☐ Attached Form PTO-1449 listing documents  
 b. ☐ Attached copies of documents listed on Form PTO-1449  
 c. ☒ A concise explanation of relevance of ISR references is given in the ISR.
19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.
20. ☐ Copy of Power to IA agent.
21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): \_\_\_\_ sheet(s) per set: ☐ 1 set informal;  
☐ Formal of size ☐ A4 ☐ 11"
22. Small Entity Status ☐ is **Not** claimed ☒ is claimed (pre-filing confirmation required)
- 22(a) \_\_\_\_\_ (No.) Small Entity Statement(s) enclosed (since 9/8/00 Small Entity Statements(s) not essential to make claim)
23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) Great Britain of:
- |     | Application No. | Filing Date    |     | Application No. | Filing Date |
|-----|-----------------|----------------|-----|-----------------|-------------|
| (1) | 9821393.7       | 1 October 1998 | (2) |                 |             |
| (3) |                 |                | (4) |                 |             |
| (5) |                 |                | (6) |                 |             |
- a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
- b. ☐ Copy of Form PCT/IB/304 attached.
24. Attached:

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25. Preliminary Amendment:

25.5 Per Item 17.c.2, cancel original pages #\_\_\_\_, claims #\_\_\_\_, Drawing Sheets #

26. Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:

Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☐ 17, ☐ 25, ☐ 25.5 (hilitte)

Total Effective Claims	17	minus 20 =	0	x \$18/\$9	=	\$0	966/967
Independent Claims	3	minus 3 =	0	x \$80/\$40	=	\$0	964/965
If any proper (ignore improper) Multiple Dependent claim is present,				add \$270/\$135	+	+135	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ BASIC FEE REQUIRED, NOW →→→→

A. If country code letters in item 1 are not "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

See item 16 re:

1. Search Report was <u>not</u> prepared by EPO or JPO -----	add \$1000/\$500	960/961
2. Search Report was prepared by EPO or JPO -----	add \$860/\$430	970/971
	+430	

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

(X) → <input type="checkbox"/> B. If <u>USPTO</u> did not issue <u>both</u> International Search Report (ISR) <u>and</u> (if box 4(b) above is X'd) the International Examination Report (IPER), -----	add \$970/\$485	+0	960/961
(only) → <input type="checkbox"/> C. If <u>USPTO</u> issued ISR but not IPER (or box 4(a) above is X'd), -----	add \$710/\$355	+0	958/959
(one) → <input type="checkbox"/> D. If <u>USPTO</u> issued IPER but IPER Sec. V boxes <u>not all</u> 3 YES, -----	add \$690/\$345	+0	956/957
(these) → <input type="checkbox"/> E. If international preliminary examination fee was paid to <u>USPTO</u> and Rules 492(a)(4) and 496(b) <u>satisfied</u> (IPER Sec. V <u>all</u> 3 boxes YES for <u>all</u> claims), -----	add \$100/\$50	+0	962/963

27. SUBTOTAL = \$565

28. If Assignment box 19 above is X'd, add Assignment Recording fee of ----\$40 +0 (581)

29. Attached is a check to cover the ----- TOTAL FEES \$565

Our Deposit Account No. 03-3975

Our Order No. 068800

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C#

M#

**CHARGE STATEMENT:** The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed

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**NOTE:** File in duplicate with 2 postcard receipts (PAT-103) & attachments.

CHARACTERISING POLYPEPTIDES THROUGH CLEAVAGE AND MASS  
SPECTROMETRY

This invention relates to a method for characterising polypeptides using mass spectrometry to identify the terminal fragments of the polypeptides. The method involves cleaving the polypeptides and isolating a single terminal peptide from each protein in a population. This invention further relates to the use of the above methods in methods of determining the expression of proteins in a tissue, cell type, or sub-cellular compartment or in analysing large protein complexes.

Techniques for profiling proteins, that is to say cataloguing the identities and quantities of proteins in a tissue, are not well developed in terms of automation or high throughput. The classical method of profiling a population of proteins is by two-dimensional electrophoresis (R.A. Van Bogelen., E.R. Olson, "Application of two-dimensional protein gels in biotechnology.", Biotechnol. Annu. Rev., 1:69-103, 1995). In this method a protein sample extracted from a biological sample is separated on a narrow gel strip. This first separation usually separates proteins on the basis of their iso-electric point. The entire gel strip is then laid against one edge of a rectangular gel. The separated proteins in the strip are then electrophoretically separated in the second gel on the basis of their size. This technology is slow and very difficult to automate. It is also relatively insensitive in its simplest incarnations.

A number of improvements have been made to increase resolution of proteins by 2-D gel electrophoresis and to improve the sensitivity of the system. One method to improve the sensitivity of 2-D gel electrophoresis and its resolution is to analyse the protein in specific spots on the gel by mass spectrometry (Jungblut P, Thiede B. "Protein identification from 2-DE gels by MALDI mass spectrometry." Mass Spectrom. Rev. 16:145-162, 1997). One such method is in-gel tryptic digestion followed by analysis of the tryptic fragments by mass spectrometry to generate a peptide mass fingerprint. If sequence information is required, tandem mass spectrometry analysis can be performed.

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More recently attempts have been made to exploit mass spectrometry to analyse whole proteins that have been fractionated by liquid chromatography or capillary electrophoresis (Dolnik V. "Capillary zone electrophoresis of proteins.", Electrophoresis 18, pp 2353-2361, 1997). In-line systems exploiting capillary electrophoresis mass spectrometry have been tested. The analysis of whole proteins by mass spectrometry, however, suffers from a number of difficulties. The first difficulty is the analysis of the complex mass spectra resulting from multiple ionisation states accessible by individual proteins. The second major disadvantage is that the mass resolution of mass spectrometers is at present quite poor for high molecular weight species, i.e. for ions that are greater than about 4 kilodaltons in mass. Thus, resolving proteins that are close in mass is difficult. A third disadvantage is that further analysis of whole proteins by tandem mass spectrometry is difficult as the fragmentation patterns for whole proteins are extremely complex and difficult to interpret.

WO 98/32876 discloses methods for profiling a population of proteins by isolating a single peptide from each protein in the population. The method disclosed in this application comprises the following steps:

1. Capturing a population of proteins onto a solid phase support by one terminus of each protein in the population.
2. Cleaving the captured proteins with a sequence specific cleavage agent.
3. Washing away peptides generated by the cleavage agent not retained on the solid phase support.
4. Releasing the terminal peptides retained on the solid phase support.
5. Analysing the released terminal peptides preferably identifying and quantifying each peptide in the mixture. The analysis is preferably performed by mass spectrometry.

In this application, the C-terminus is indicated to be more preferable as the terminus by which to capture a population of proteins, since the N-terminus is often blocked. In order to capture a population of proteins by the C-terminus, the C-terminal carboxyl group must be distinguished from other reactive groups on a protein and must be reacted specifically

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with a reagent that can effect immobilisation. In many C-terminal sequencing chemistries the C-terminal carboxyl group is activated to promote formation of an oxazolone group at the C-terminus. During the activation of the C-terminal carboxyl, side chain carboxyls are also activated but these cannot form an oxazolone group. It has been reported that the C-terminal oxazolone is less reactive to nucleophiles under basic conditions than the activated side-chain carboxyls, offering a method of selectively capping the side chain carboxyl groups (V.L. Boyd et al., *Methods in Protein Structure Analysis*: 109-118, Plenum Press, Edited M.Z. Atassi and E. Appella, 1995). Other more reactive side chains can be capped prior to the activation of the carboxyls using a variety of conventional reagents. In this way all reactive side chains can be capped and the C-terminus can be specifically labelled.

EP-A-0 594 164 describes a method of isolating a C-terminal peptide from a protein in a method to allow sequencing of the C-terminal peptide using N-terminal sequencing reagents. In this method the protein of interest is digested with an endoprotease which cleaves at the C-terminal side of lysine residues. The resultant peptides are reacted with DITC polystyrene which reacts with all free amino groups. N-terminal amino groups that have reacted with the DITC polystyrene can be cleaved with trifluoroacetic acid (TFA) thus releasing the N-terminus of all peptides. The epsilon amino group of lysine is not cleaved, however, and all non-terminal peptide are thus retained on the support and only C-terminal peptides are released. According to this patent the C-terminal peptides are recovered for micro-sequencing.

However, none of the above methods is capable of readily identifying all of the proteins present in a population, and in fact many of these methods cannot uniquely identify all of the proteins present at all.

Accordingly, it is an object of the present invention to overcome the problems associated with the prior art, and to provide a method for characterising polypeptides which is capable of uniquely identifying a greater proportion of proteins present in a sample, than is possible using prior art methods. It is thus an object of the present invention to provide

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improved methods for isolating a single terminal peptide from each protein in a mixture, complex or population (prepared in an arbitrary fashion). A further object of this invention is to provide such methods that are amenable to automation. It is also an object of this invention to provide methods to improve the sensitivity of the mass spectrometry analysis of terminal peptides generated by the methods of this invention.

Accordingly the present invention provides a method for characterising a polypeptide or a population of polypeptides, which method comprises:

- (a) contacting a sample comprising one or more polypeptides with a first cleavage agent to generate polypeptide fragments;
- (b) isolating one or more polypeptide fragments, each fragment comprising the N-terminus or the C-terminus of the polypeptide from which it was fragmented;
- (c) identifying the isolated fragments by mass spectrometry;
- (d) repeating steps (a)-(c) on the sample using a second cleavage agent that cleaves at a different site from the first cleavage agent; and
- (e) characterising the one or more polypeptides in the sample from the fragments identified in steps (c) and (d).

In the context of the present invention a polypeptide includes any peptide comprising two or more amino acids, and includes any protein. In the present context, the sample used in the repeat step (d) is the same sample as that used in step (a). The "same sample" may mean (for example) one of a plurality of portions separated from one original sample, there being one portion for each repetition of the steps (a)-(c).

The steps (a)-(c) can be repeated any number of times, e.g. once, twice or more times. For each repetition a cleavage agent is used which cleaves at a different site than the previous cleavage agents used. This gives a different set of terminal fragments for each repetition, even though the same sample is being treated. If two or more proteins cannot be distinguished by means of their terminal fragments produced by cleavage at a particular site (e.g. if two or more proteins have terminal fragments sharing the same or

indistinguishable mass) then cleavage at a different site may (for the same two or more proteins) produce terminal fragments of different masses. Thus the resolution of the method is enhanced by repetition using a plurality of different cleavage agents.

The cleavage agents used in the present invention are not especially limited. Preferred cleavage agents include an endopeptidase or a chemical cleavage agent. More preferably the cleavage agent employed comprises Lys-C endopeptidase, a thiocyanate compound, cyanogen bromide, BNPS-skatole, trypsin, chymotrypsin, and/or thrombin.

The present invention also provides a method for characterising a polypeptide or a population of polypeptides, which method comprises:

- (f) contacting a sample comprising one or more polypeptides with a first capping agent in a first capping step to introduce capping groups on one or more reactive side chains of the polypeptides;
- (g) contacting the sample with a cleavage agent to generate polypeptide fragments;
- (h) isolating one or more polypeptide fragments, each fragment comprising the N-terminus or the C-terminus of the polypeptide from which it was fragmented;
- (j) identifying the isolated fragments by mass spectrometry;
- (k) repeating steps (f)-(j) on the sample using a second capping agent that introduces capping groups at the same side chains as the first capping step, but uses capping groups having different mass than the capping groups used in the first capping step; and
- (l) characterising the one or more polypeptides in the sample from the fragments identified in steps (j) and (k).

The method may use the same cleavage agent or different cleavage agents for each repetition, as long as capping groups having different masses are employed for each repetition. The steps (f)-(j) can be repeated any number of times, e.g. once, twice or more times. For each repetition a capping group is used which has a different mass than the corresponding previous capping group that was used. This gives a different set of terminal fragments for each repetition, even though the same sample is being treated with



the same cleavage agent. If two or more proteins cannot be distinguished from their terminal fragments capped with a particular capping group or groups (e.g. if two or more proteins have terminal fragments sharing the same or indistinguishable mass) then use of one or more capping groups having a different mass to the corresponding groups used in the previous capping step may (for the same two or more proteins) produce terminal fragments of different masses. Thus the resolution of the method is enhanced by repetition using a plurality of capping groups, each having a different mass.

In the above-described methods, when the side chains of the proteins in the population are to be capped, the side chains to be capped may comprise one or more of the following:

- the NH<sub>2</sub> side chain in arginine;
- the NH<sub>2</sub> side chain in asparagine;
- the NH<sub>2</sub> side chain in glutamine;
- the NH<sub>2</sub> side chain in lysine;
- the COOH side chain in aspartic acid;
- the COOH side chain in glutamic acid;
- the OH side chain in serine;
- the OH side chain in threonine;
- the OH side chain in thyroxine;
- the OH side chain in tyrosine; and
- the SH side chain in cysteine.

The capping agents used in the present invention are not especially limited. Preferred capping agents include iodoacetate compounds, isocyanate compounds (e.g. phenyl isocyanate), silyl compounds (e.g. trimethylchlorosilane), anhydride compounds (e.g. acetic anhydride and trimethylacetic anhydride), vinylsulphone compounds (e.g. phenyl vinylsulphone and methyl vinylsulphone) and vinyl pyridine derivatives (e.g. 4-vinyl pyridine). The mass of the capping group can be altered by substitution. Such substitution is not particularly limited, provided that the capping reaction is still able to proceed. Substitution with deuterium or a halogen such as an iodine group is preferred.

Thus, in one preferred aspect, this invention provides a method of generating a protein expression profile with improved resolution by combining two or more protein expression profiles where the profiles have been generated from the same protein mixture but which have been treated with two or more different sequence specific cleavage agents. A different sequence specific cleavage agent being used in the generation of each individual profile. In this context resolution refers to the proportion of proteins in a population that can be identified uniquely from a database on the basis of their terminal peptide mass alone.

In a further preferred aspect this invention provides a method of generating a protein expression profile with improved resolution by combining two or more expression profiles where the profiles have been generated from the same protein mixture and have been cleaved with the same sequence specific cleavage reagent but where specific side chains, such as amino acid side chains, have been capped with two or more capping reagents with different masses. A different set of capping agents is used in the generation of each individual profile.

More preferred aspects of the invention will now be discussed in detail. In a first preferred aspect, this invention provides a method of generating a population of terminal peptides comprising the steps of:

1. Immobilising a population of proteins onto a solid support.
2. Contacting the immobilised population of proteins with a reagent which reacts with the alpha-amino group at the N-terminus of the proteins and with any lysine epsilon-amino groups. This reagent may optionally also react with any serine and threonine side chains in order to cap them. In this context capping means reacting the side-chains with a reagent which will render the side-chains unreactive to any other reagents used in the subsequent steps of this method. If the reagent does not cap side chains other than amine groups additional capping agents may be applied to cap other reactive side chains so that substantially all reactive side-chains are capped.

3. Contacting the protein population with a reagent that will 'activate' the free carboxyl groups of the proteins. The activation reagent should preferably promote the formation of an oxazolone group at the C-terminal activated carboxyls of the proteins in the population.
4. Contacting the resultant derivitised proteins with a nucleophile under basic conditions to cap the activated side chain carboxyl derivatives. The C-terminal oxazolinone is less reactive to nucleophiles than the activated side chain carboxyls. In this way all reactive side chain functionalities are capped. Preferably the nucleophile is added as a thiocyanate salt.
5. Contacting the reaction mixture with an appropriate acid, preferably TFA, which promotes reaction of the C-terminal oxazolone group with thiocyanate to give a C-terminal thiohydantoin derivative.
6. Contacting the proteins with a cleavage agent to cleave the C-terminal thiohydantoin from the derivitised proteins to expose the carboxyl of the penultimate amino acid in each protein.
7. Contacting the exposed penultimate carboxyl with an appropriate reagent to permit solid phase capture of proteins by the C-terminus.
8. Contacting the C-terminally modified proteins with a sequence specific cleavage agent.
9. Capturing the C-terminal peptides onto a solid phase support and washing away the non-terminal peptides.
10. Optionally reacting the terminal amine group of the captured peptides with a mass spectrometry sensitisation reagent.
11. Releasing the captured C-terminal peptides from the solid phase support.
12. Recovering the released peptides.
13. Analysing the C-terminal peptides by mass spectrometry.

Any one of the above preferred steps, or any combination of the above preferred steps can be utilised in the general methods of the invention as already described above.

In a second preferred aspect this invention provides a method of generating a population of terminal peptides comprising the steps of:

1. Immobilising a population of proteins onto a solid phase support.
2. Contacting the immobilised population of proteins with a reagent which reacts with the alpha-amino group at the N-terminus of the proteins and with any lysine epsilon-amino groups. This reagent may optionally also react with any serine and threonine side chains in order to cap them. In this context capping means reacting the side-chains with a reagent which will render the side-chains unreactive to any other reagents used in the subsequent steps of this method. If the reagent does not cap side chains other than amine groups additional capping agents may be applied to cap other reactive side chains so that substantially all reactive side-chains are capped.
3. Contacting the protein population with a reagent that will 'activate' the free carboxyl groups of the proteins. The activation reagent should preferably promote the formation of an oxazolone group at the C-terminal activated carboxyls of the proteins in the population.
4. Contacting the resultant derivitised proteins with a nucleophile under basic conditions to cap the activated side chain carboxyl derivatives. The C-terminal oxazolinone is less reactive to nucleophiles than the activated side chain carboxyls. In this way all reactive side chain functionalities are capped. Preferably the nucleophile is added as a salt with an inert anion.
5. Hydrolysing the C-terminal oxazolone to regenerate the C-terminal carboxyl species.
6. Contacting the proteins with an activation agent to activate the C-terminal carboxyl group
7. Contacting the activated terminal carboxyl group with an appropriate reagent to permit solid phase capture of proteins by the C-terminus.
8. Contacting the C-terminally modified proteins with a sequence specific cleavage agent.
9. Capturing the C-terminal peptides onto a solid phase support and washing away the non-terminal peptides.

10. Optionally reacting the terminal amine group of the captured peptides with a mass spectrometry sensitisation reagent.
11. Releasing the captured C-terminal peptides from the solid phase support.
12. Recovering the released peptides.
13. Analysing the C-terminal peptides by mass spectrometry.

Any one of the above preferred steps, or any combination of the above preferred steps can be utilised in the general methods of the invention as already described above.

In a third preferred aspect, this invention provides a method of generating a population of terminal peptides from a population of proteins comprising the steps of:

1. Digesting a population of proteins completely with a Lys-C specific cleavage enzyme, i.e. a reagent that cuts at the peptide bond immediately agent to a lysine residue on the C-terminal side of that residue.
2. Contacting the resultant peptides with an activated solid support that will react with free amino groups.
3. Contacting the captured peptides with a reagent that which cleaves at the alpha amino groups of each peptide on the support. All peptides that are not C-terminal will have a lysine residue covalently linking them to the solid support. Thus free C-terminal peptides are selectively released.
4. Recovering the released peptides.
5. Optionally contacting the released peptide with reagents to cap reactive side chains.
6. Optionally reacting the terminal amine group of the captured peptides with a mass spectrometry sensitisation reagent.
7. Analysing the peptides by mass spectrometry.

Any one of the above preferred steps, or any combination of the above preferred steps can be utilised in the general methods of the invention as already described above.

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In order that only the fragments containing lysine residues remain bound to the solid support via two amino groups, (so that all fragments except those containing lysine groups are released from the solid support during the releasing step) the fragments are preferably attached to the solid phase at a pH at which the side chain  $\text{NH}_2$  of lysine is not protonated. Thus, this step is preferably carried out at a pH of from 11-11.5.

### *Generation of a population of C-terminal peptides*

#### *The use of C-terminal sequencing reagents*

Various preferred embodiments of the above aspects of this invention are discussed below.

In the first step of the first and second preferred aspects of this invention, a population of proteins is immobilised onto a solid phase support, preferably non-covalently. Zitex (porous Teflon from Norton Performance Plastics, Wayne, NJ) membranes can be used to effect non-covalent immobilisation of proteins on a solid phase support (Bailey et al., "Automated carboxy-terminal sequence analysis of peptides and proteins using diphenyl phosphorothio-cyanatidate", Protein Science 1: 1622 - 1633, 1992; Bailey et al., Anal. Biochem. 212: 366-374, 1993.) Polyvinylidenedifluoride membranes (Millipore) can also be used to immobilise proteins.

Step 2 of the first and second preferred aspects of this invention involves capping of the reactive side chains of a population of proteins. It is well known in the art that the reactive side-chain functionalities can be selectively capped. Reactive side-chains include lysine, serine, threonine, tyrosine and cysteine. Cysteine is often cross-linked with itself to form disulphide bridges. For the purposes of this invention it is preferred that these bridges are broken. This can be effected by reducing the disulphide bridge to a pair of thiols with mercaptoethanol. Thiols can be selectively capped by iodacetate (Aldrich) under mildly basic conditions which promote the formation of a thiolate ion (Mol. Microbiol 5: 2293, 1991). An appropriate mild base would be a carbonate. In other embodiments the population of proteins may be treated with an isocyanate compound. Isocyanates will react almost exclusively with the alpha-amino group at the N-terminus of the proteins and

with any lysine epsilon-amino groups, i.e. with primary amines under mild conditions, i.e. at room temperature in a neutral solvent to give a urea derivative. These reagents can also be made to react with any hydroxyl bearing side-chains, such as serine, threonine and tyrosine side chains, in the presence of an appropriate catalyst such as pyridine or a tin compound, such as dibutylstanyl laurate, to give a urethane derivative. In an alternative embodiment the population of proteins can be treated with a silyl compound such as trimethylchlorosilane (Sigma). These compounds react readily with most reactive functional groups. Amine derivatives are not stable under aqueous conditions and so can be hydrolysed back to the free amine if that is desired. The above examples are intended to illustrate methods of capping reactive side-chain functionalities and are not intended to limit the scope of this invention. A wide variety of protective groups are known in the art and it is envisaged that a large proportion of these could be used to complete the steps of this invention.

In step 3 of the first and second preferred aspects of this invention the carboxyl side chains are then 'activated'. Acetic anhydride has been widely used for this purpose, which generates mixed anhydrides at carboxyl groups. In some embodiments, step 2 of first and second preferred aspects of this invention may be combined with step 3. Activation of side chain carboxyls with an anhydride compound also results in capping of reactive side chains, such as lysine, serine, threonine and tyrosine. In embodiments where step 2 and 3 are combined, it is preferred that the activation reagent chosen is stable as a capping agent for the reactive side chains, as some anhydride derivatives are not stable under basic or acidic conditions. Trimethylacetic anhydride is more stable under these conditions through steric effects, for example. An alternative, more preferred, activation reagent is Woodward's Reagent K (N-Ethyl-5-phenylisoxazolium-3-sulphonate available from Sigma). In another preferred embodiment of this aspect of the invention, activation is achieved by treatment with a reactive phosphate such as tetraphenyl pyrophosphate (Aldrich) or diphenyl phosphochloridate. According to US patent 5,665,603, the activation step required in peptide sequencing methods to generate a carboxyl derivative at the C-terminus of a peptide or poly-peptide which can react to form a thiohydantoin can be effected more efficiently under milder conditions with acyl phosphate compounds,

such as diphenyl phosphochloridate or tetraphenyl pyrophosphate (Aldrich). The C-terminal carboxyl is reacted with the reactive phosphate in the presence of a base to deprotonate the carboxyl group. Preferred bases are tri-ethylamine, diisopropylethylamine or pyridines, i.e. a base which won't react with the resultant acyl phosphates. The phosphorylation reaction to activate the C-terminal carboxyl preferably uses equimolar quantities of reactive phosphate and base. These two reagents are added in large excess to a poly-peptide in a polar, aprotic solvent, e.g. acetonitrile (ACN), dimethylformamide or an ether, preferably ACN. The reaction is typically complete in 5 to 10 minutes and usually less than 30 minutes at room temperature. Temperature can vary: the reactions are performed at 55°C in an automated sequencer in line with other reactions taking place. The C-terminal activated derivatives of carboxyl groups will spontaneously cyclise to form an oxazolone intermediate whilst the side chain carboxyls remain activated.

The activated side-chain carboxyl derivatives apparently react with nucleophiles under basic conditions whilst the C-terminal oxazolone group is much less reactive to nucleophiles (V.L. Boyd et al., *Methods in Protein Structure Analysis*: 109 - 118, Plenum Press, Edited M.Z. Atassi and E. Appella, 1995). Hence, in step four of the first and second preferred aspects of this invention the protein population is contacted with a nucleophile under basic conditions. A preferred nucleophile is a primary amine compound. If amine nucleophiles are used the side chains can be amidated. Preferred amine nucleophiles are piperidine, methylamine, ethylamine or other amine, added with an appropriate base. To perform the amidation reaction equimolar quantities of base and nucleophile are added in a polar aprotic solvent, preferably acetonitrile, with the reagents in a large excess over the mixture of proteins. Ammonia generates asparagine from activated aspartic acid residues and glutamine from glutamic acid residues. This may reduce the information in the peptide's mass and ammonia may not be a preferred nucleophile. In an alternative embodiment an alcohol can be used to esterify the activated side-chain carboxyl derivatives under acidic conditions. An appropriate alcohol, e.g. methanol, can be added in an appropriate, anhydrous acidic solvent, e.g. TFA.



In the first preferred aspect of this invention the nucleophile may be added as a thiocyanate salt. The oxazolone reacts under acidic conditions with isothiocyanate and so after an initial period under basic conditions to promote amidation the reaction is acidified with trifluoroacetic acid to promote C-terminal thiohydantoin formation.

In the second preferred aspect of this invention, the amine nucleophile may be added as a salt with an inert group, e.g. a carbonate. Preferably a volatile salt is used to facilitate removal of unused reagent. In this way the oxazolone can be prevented from reacting so that the oxazolone can be hydrolysed to regenerate a free carboxyl at the C-terminus. In a further embodiment the amine nucleophile can be added as a salt with a biotinylation agent with an appropriate functionality to react with the oxazolone.

In the first aspect of this invention where the nucleophile is added as a thiocyanate salt, the reaction mixture is then acidified with an appropriate acid, preferably TFA which promotes reaction of the C-terminal oxazolone group with thiocyanate to give a C-terminal thiohydantoin derivative. The C-terminal thiohydantoin is then cleaved from the derivitised proteins to expose the carboxyl of the penultimate amino acid in each protein. Cleavage of the thiohydantoin from the C-terminal can be effected by a variety of methods including hydrolysis by acid or base. More preferably cleavage is effected by a reagent such as sodium trimethylsilanolate in an alcoholic solvent (J.M. Bailey et al., Protein Science 1: 68 - 80, 1992).

In step 7 of the first and second preferred aspects of this invention the exposed terminal carboxyl may then be reacted with an appropriate reagent to permit solid phase capture of proteins by the C-terminus. A reagent such as biotinamidocaproyl hydrazide is reactive with free carboxylic acids. A reagent such as 5-(biotinamido)pentylamine can be used allowing capture by avidin. Prior to reaction with this biotinylation reagent, free carboxyl termini must be activated. A variety of activation agents can be used, but should preferably not promote the formation of an oxazolone. An anhydride compound that is sterically hindered might be appropriate. In an alternative embodiment the activated terminal carboxyl can be reacted with a solid phase support functionalised with an

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appropriate group to react with the activated carboxyl. A free amine group would be appropriate. To permit selective release the carboxyl reactive functionality should be linked to the support by a cleavable linker. A variety of cleavable linkers are known in the art. Photocleavable linkers are well known in the art (Lloyd-Williams et al., Tetrahedron 49: 11065 - 11133, 1993). There are also numerous chemically cleavable linkers, e.g. thioesters may be cleaved by hydroxyl-amine.

In step 8 of the first and second preferred aspects of this invention the C-terminally modified proteins are then treated with a sequence specific cleavage agent. Preferred cleavage agents are chemical reagents which are volatile permitting easy removal of unreacted reagent. Appropriate chemical cleavage reagents include cyanogen bromide which cleaves at methionine residues and BNPS-skatole which cleaves at tryptophan residues (D.L. Crimmins et al., Anal. Biochem. 187: 27-38, 1990). In other embodiments sequence specific endoproteinases such as trypsin, chymotrypsin, thrombin or other enzymes may be used.

In step 9 of the first and second preferred aspects of this invention the terminally modified peptides are captured on a solid phase support. In embodiments where the terminal carboxyl is biotinylated the C-terminal peptides may then be captured onto a solid phase support derivitised with streptavidin. The non-terminal peptides can then be washed away.

In step 10 of the first and second preferred aspects of this invention the captured C-terminal peptides are optionally reacted with a mass spectrometry sensitisation reagent. The ion detectors of a mass spectrometer are extremely sensitive, and can detect the arrival of single ions. Thus detection of ions is not the limiting factor that determines the sensitivity of a mass spectrometer. Generally, the most limiting factor is the ionisation of the analyte. In a typical electrospray source or FAB source, only one in a thousand molecules of analyte will actually ionise and be detected. For the purposes of improving this process, it is desirable to introduce a sensitisation compound into the peptides for detection that will pre-ionise the peptide. Preferred compounds include quaternary

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ammonium ions or metal ion chelation agents. An exemplary compound might be 4-(3-pyridylmethylaminocarboxypropyl)phenyl isocyanate (E.J. Bures et al., "Synthesis and evaluation of a panel of novel reagents for stepwise degradation of polypeptides", Methods in Protein Structure Analysis, Plenum Press, New York, 1995 disclose the use of the isothiocyanate form of this compound as a mass spectrometry sensitiser). The introduction of a sensitisation group not only improves sensitivity but also reduces the risk of competition for ionisation by analyte molecules. This means that all peptides should be more evenly represented in the mass spectrum.

In step 11 of the first and second preferred aspects of this invention the captured C-terminal peptides are then released from the solid phase support. In embodiments where the terminal carboxyl is biotinylated, the avidin captured peptides can be released by treatment with acid. Preferably TFA is used to facilitate recovery of the released peptides as this is volatile and can be readily evaporated to permit recovery of the peptides.

In step 12 of the first and second preferred aspects of this invention the released peptides are recovered. In step 13 of the third and fourth aspects of this invention the peptides are analysed by mass spectrometry. In one embodiment the peptides are embedded in a MALDI matrix, such as cinammic acid, on an appropriate support and are analysed by MALDI mass spectrometry to determine a peptide mass fingerprint for the population of C-terminal peptides. MALDI is a preferred analysis technique as this ionisation technique favours the formation of  $[M+H]^+$  ions. Thus there is usually only one major peak in the mass spectrum for each peptide.

In a further embodiment the recovered peptides may be dissolved in an appropriate solvent and can be analysed by a spraying inlet system such as electrospray ionisation mass spectrometry. Similarly Fast Atom Bombardment (FAB) and related interfaces may be used. This may include in-line liquid chromatography separation of peptides prior to analysis by mass spectrometry. Capillary electrophoresis may be used, or HPLC or capillary iso-electric focusing. Dynamic FAB is a particularly preferred method as this

method of ionisation promotes the generation of ions in the form in which they exist in the matrix used to introduce them into the mass spectrometer. This means that the ions present in the mass spectrum ought to be the same as they are in solution if a liquid matrix is used. Since in the present invention reactive side chains may be capped and a mass spectrometry sensitisation agent may be introduced into the peptides to be analysed, it is possible to ensure that all peptides have only a single charge in solution which is represented as a single mass peak in the final mass spectrum of a peptide population. Tandem mass spectrometers can be coupled to a spraying interface or to a FAB interface. Tandem mass spectrometry permits sequence information to be determined for a peptide and permits identification of covalent modifications of the protein.

#### *The use of DITC glass*

Various embodiments of the third preferred aspect of this invention are discussed here.

In step 1 of the third preferred aspect of this invention a population of proteins is completely digested with a Lys-C specific cleavage enzyme, e.g. endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim).

In step 2 of the third preferred aspect of this invention the resultant peptide are contacted with a solid support which reacts with amine. In one embodiment the peptide population is reacted with Isothiocyanato glass (DITC glass, Sigma) in the presence of a base. This captures all peptides to the support through any free amino groups.

-- In step 3 of the third preferred aspect of this invention the captured peptides are contacted with a reagent that which cleaves at the alpha amino groups of each peptide on the support. In embodiments where DITC glass is used the peptides are treated with an appropriate volatile acid (TFA) which cleaves the N-terminal amino acid from each peptide on the support. All peptides that are not C-terminal will have a lysine residue covalently linking them to the solid support. Thus free C-terminal peptides are selectively released.

In step 4 of the third preferred aspect of this invention the released peptides can be recovered from the TFA by evaporating the TFA solvent used to cleave the peptides from the support.

In step 5 of the third preferred aspect of this invention the released peptides may be contacted with reagents to cap reactive side chains. Appropriate reagents include those discussed above.

In step 6 of the third preferred aspect of this invention the peptides are optionally reacted with a mass spectrometry sensitisation reagent. See the discussion above regarding step 10 of the first and second preferred aspects of this invention.

In step 7 of the third preferred aspect of this invention the peptides are analysed by mass spectrometry. In one embodiment the terminal peptides are embedded in a MALDI matrix such as cinammic acid and are analysed by MALDI mass spectrometry to determine a peptide mass fingerprint for the population of C-terminal peptides.

As in the other preferred aspects of this invention, alternative methods of analysing peptides by mass spectrometry can be used. Thus in alternative embodiments the recovered peptides may be dissolved in an appropriate solvent and can be analysed by a spraying inlet system such as electrospray ionisation mass spectrometry. Similarly Fast Atom Bombardment and related interfaces may be used. This may also include in-line liquid chromatography separation prior to mass spectrometry. Tandem mass spectrometers can be coupled to a spraying interface or to a FAB interface. Tandem mass spectrometry permits sequence information to be determined for a peptide and permits identification of covalent modifications of the protein.

## Examples

A series of short computer programs (written in PERL) were employed to analyse the SWISSPROT public domain database of protein sequences to determine, for a given

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organism, what proportion of proteins could be identified uniquely on the basis of their terminal peptide masses alone. Proteins were extracted from the SWISSPROT database release 35. Analyses were performed on data from the H. influenzae genome. The genome of this organism has been completely sequenced and nearly all predicted open reading frames have been identified. There were 1882 H. influenzae proteins present in this release of the database. Profiles with cleavage at methionine by Cyanogen Bromide and cleavage at tryptophan were tested. It was assumed that the terminal amino acid is not cleaved by the profiling chemistry.

In all of the examples it has been assumed that the mass spectrometer has a mass resolution of 5000. This means that the instrument can resolve a difference in mass of 1 part in 5000, or in other words a molecule of mass 5000 daltons can be resolved from a molecule of mass 4999 daltons. It is also assumed that each peptide only gives rise to a single molecular ion peak.

*Example 1 - Cleavage with Lys-C and SCN*

In this example the 1882 H. influenzae proteins are cleaved with the endoproteinase Lys-C which cuts proteins at the C-terminal side of a Lysine residue. The peptides are then reacted with a solid phase support derivitised with phenylisothiocyanate moieties which readily react with primary amines generated at the cleavage sites and present on Lysine side chains as described above. All peptides have at least 1 lysine residue except the C-terminal peptide which has none. All peptides are captured onto the solid support by their N-terminal primary amines. All non C-terminal peptides are also captured by their lysine side chains as well. Phenylisocyanate can be induced to cleave the N-terminal residue of all peptides. All lysine residue carrying peptides will still remain attached to the support as these isothiocyanate derivatives cannot cleave. Thus C-terminal peptides are released from the solid phase support. The C-terminal peptides can then be isolated. They may be modified further if desired for reasons discussed above. After isolation of the C-terminal peptides and any additional side chain modifications the peptides may be analysed by mass spectrometry.

It is assumed that no other side chains are capped and the unmodified peptides are analysed directly.

### *Results*

1882 protein records analysed.

7 proteins did not have a cleavage site for the reagent used.

870 peptide mass(es) shared by 1 protein(s)

155 peptide mass(es) shared by 2 protein(s)

43 peptide mass(es) shared by 3 protein(s)

19 peptide mass(es) shared by 4 protein(s)

6 peptide mass(es) shared by 5 protein(s)

9 peptide mass(es) shared by 6 protein(s)

2 peptide mass(es) shared by 7 protein(s)

3 peptide mass(es) shared by 8 protein(s)

1 peptide mass(es) shared by 9 protein(s)

1 peptide mass(es) shared by 10 protein(s)

2 peptide mass(es) shared by 11 protein(s)

2 peptide mass(es) shared by 12 protein(s)

1 peptide mass(es) shared by 13 protein(s)

1 peptide mass(es) shared by 17 protein(s)

1 peptide mass(es) shared by 19 protein(s)

1 peptide mass(es) shared by 23 protein(s)

1 peptide mass(es) shared by 33 protein(s)

1 peptide mass(es) shared by 205 protein(s)

This peptide mass fingerprint identifies 46.2 % of proteins uniquely on the basis of their terminal peptide mass alone.

*Example 2 - Capping hydroxyl side-chains*

In this example the reactive hydroxyl carrying side chains of serine, threonine and tyrosine are capped with a silyl protecting group generating for example trimethylsilyl derivatives at these side chains. The side chain carboxyl groups are activated with an appropriate reagent, such as tetraphenylpyrophosphate and these are then converted to piperidine amides by reaction with piperidine in the presence of a base as described above.

*Results*

1882 protein records analysed.

7 proteins did not have a cleavage site for the reagent used.

937 peptide mass(es) shared by 1 protein(s)

149 peptide mass(es) shared by 2 protein(s)

34 peptide mass(es) shared by 3 protein(s)

17 peptide mass(es) shared by 4 protein(s)

6 peptide mass(es) shared by 5 protein(s)

8 peptide mass(es) shared by 6 protein(s)

2 peptide mass(es) shared by 7 protein(s)

4 peptide mass(es) shared by 8 protein(s)

2 peptide mass(es) shared by 10 protein(s)

1 peptide mass(es) shared by 11 protein(s)

1 peptide mass(es) shared by 12 protein(s)

1 peptide mass(es) shared by 13 protein(s)

1 peptide mass(es) shared by 17 protein(s)

1 peptide mass(es) shared by 19 protein(s)

1 peptide mass(es) shared by 23 protein(s)

1 peptide mass(es) shared by 33 protein(s)

1 peptide mass(es) shared by 205 protein(s)



This peptide mass fingerprint identifies 49.8 % of proteins uniquely on the basis of their terminal peptide mass alone.

*Example 3 - C-terminal capture and cleavage with cyanogen bromide*

In this example the 1882 H. influenzae proteins are immobilised non-covalently onto a solid phase support. The proteins are treated with trifluoroacetic anhydride. This reagent will cap the reactive side chains of lysine, serine, threonine and tyrosine to give trifluoroacetyl derivatives. This reagent will also activate side chain and terminal carboxyl groups. The C-terminal activated carboxyls spontaneously form an oxazolone. The unreacted trifluoroacetic anhydride is washed away. The activated side chains are then reacted with piperidine in the presence of a non-aqueous base which will generate piperidine amides at the activated side chain carboxyls. The C-terminal oxazolone is then hydrolysed back to the original free carboxyl which is then modified to permit capture onto a solid phase support, e.g. by biotinylation. The modified proteins are then cleaved with cyanogen bromide which cleaves at methionine residues. The resultant peptides are washed in an inert solvent and released from the solid phase support on which the chemistry is performed. The C-terminal peptides are selectively biotinylated permitting them to be captured onto an avidinated support. This allows non-terminal peptides to be washed away. The C-terminal peptides are then released from the solid support and analysed by mass spectrometry.

*Results*

1882 protein records analysed.

18 proteins did not have a cleavage site for the reagent used.

1461 peptide mass(es) shared by 1 protein(s)

157 peptide mass(es) shared by 2 protein(s)

21 peptide mass(es) shared by 3 protein(s)

4 peptide mass(es) shared by 4 protein(s)

2 peptide mass(es) shared by 5 protein(s)

3 peptide mass(es) shared by 6 protein(s)

This peptide mass fingerprint identifies 77.6 % of proteins uniquely on the basis of their terminal peptide mass alone.

*Example 4 - C-terminal capture and cleavage with BNPS-skatole*

In this example the 1882 H. influenzae proteins are treated as in example 3 except that after the C-terminal carboxyl has been modified to permit capture onto a solid phase support the proteins are cleaved with BNPS-skatole (3-bromo-3-methyl-2-(o-nitrophenylsulphenyl)indolenine) which cleaves proteins chemically at tryptophan residues rather than cyanogen bromide. The resultant peptides are then desorbed from their solid phase support and incubated with an avidinated support. This allows non-terminal peptides to be washed away. The C-terminal peptides are then released from the solid support and analysed by mass spectrometry. In this example, therefore, the reactive side chains of lysine, serine, threonine and tyrosine are trifluoroacetyl derivatives again and side chain carboxyls are piperidine amide derivatives.

*Results*

1882 protein records analysed.

361 proteins did not have a cleavage site for the reagent used.

1484 peptide mass(es) shared by 1 protein(s)

163 peptide mass(es) shared by 2 protein(s)

15 peptide mass(es) shared by 3 protein(s)

5 peptide mass(es) shared by 4 protein(s)

1 peptide mass(es) shared by 7 protein(s)

This peptide mass fingerprint identifies 78.9 % of proteins uniquely on the basis of their terminal peptide mass alone.

*Example 5 - Combining two profiles*

The list of unique proteins generated in example 3 where cleavage Cyanogen Bromide was used and in the example 4 where cleavage by BNPS-skatole was used were combined to determine the sum of proteins that were identified uniquely on the basis of their mass alone between the two profiles.

1774 proteins are uniquely resolved between the two profiles. This amounts to resolving 94.3 % of the H. influenzae proteins in the SWISSPROT database uniquely on the basis of terminal peptide masses alone. This is a 19.5 % improvement in resolution over the better individual profile.

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## CLAIMS:

1. A method for characterising a population of polypeptides, which method comprises:
- (a) contacting a sample comprising polypeptides with a first cleavage agent to generate polypeptide fragments;
  - (b) isolating one or more polypeptide fragments, each fragment comprising the N-terminus or the C-terminus of the polypeptide from which it was fragmented;
  - (c) identifying the isolated fragments by mass spectrometry;
  - (d) repeating steps (a)-(c) on the sample using a second cleavage agent that cleaves at a different site from the first cleavage agent; and
  - (e) characterising the polypeptides in the sample from the fragments identified in steps (c) and (d).
2. A method according to claim 1, wherein the step (d) comprises repeating steps (a)-(c) two or more times, each time using a further cleavage agent that cleaves at a different site from the previous cleavage agents.
3. A method according to claim 1 or claim 2, comprising a further capping step prior to step (a), which capping step comprises reacting the polypeptides in the sample with one or more capping agents to introduce capping groups on one or more reactive side chains of the polypeptides.
4. A method according to claim 3, wherein the capping step and steps (a)-(c) are repeated one, two, or more times, each time introducing capping groups at the same side chains as the previous capping steps, but using capping groups having different mass than the corresponding capping groups used in the previous capping steps.

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5. A method for characterising a polypeptide or a population of polypeptides, which method comprises:

- (f) contacting a sample comprising one or more polypeptides with a first capping agent in a first capping step to introduce capping groups on one or more reactive side chains of the polypeptides;
- (g) contacting the sample with a cleavage agent to generate polypeptide fragments;
- (h) isolating one or more polypeptide fragments, each fragment comprising the N-terminus or the C-terminus of the polypeptide from which it was fragmented;
- (j) identifying the isolated fragments by mass spectrometry;
- (k) repeating steps (f)-(j) on the sample using a second capping agent that introduces capping groups at the same side chains as the first capping step, but uses capping groups having different mass than the capping groups used in the first capping step; and
- (l) characterising the one or more polypeptides in the sample from the fragments identified in steps (j) and (k).

6. A method according to claim 5, wherein the steps (f)-(j) are repeated two or more times, each time introducing capping groups at the same side chains as the previous capping steps, but using capping groups having different mass than the corresponding capping groups used in the previous capping steps.

7. A method according to claim 5 or claim 6, wherein the step (k) comprises repeating steps (f)-(j) one, two, or more times, each time using a further cleavage agent that cleaves at a different site from the previous cleavage agents.

8. A method according to any of claims 3-7, wherein the side chains to be capped comprise one or more of the following:
- the  $\text{NH}_2$  side chain in arginine;
  - the  $\text{NH}_2$  side chain in asparagine;
  - the  $\text{NH}_2$  side chain in glutamine;
  - the  $\text{NH}_2$  side chain in lysine;
  - the  $\text{COOH}$  side chain in aspartic acid;
  - the  $\text{COOH}$  side chain in glutamic acid;
  - the  $\text{OH}$  side chain in serine;
  - the  $\text{OH}$  side chain in threonine;
  - the  $\text{OH}$  side chain in thyroxine;
  - the  $\text{OH}$  side chain in tyrosine; and
  - the  $\text{SH}$  side chain in cysteine.
9. A method according to any preceding claim, wherein the fragments are isolated by capture on a solid phase, such as DITC glass or polystyrene isothiocyanate.
10. A method according to claim 9, wherein the capture involves covalently bonding the fragments to the solid phase.
11. A method according to claim 10, wherein the fragments are bound to the solid phase through their N-termini.
12. A method according to any preceding claim, wherein each isolated fragment comprises the C-terminus of the polypeptide from which it was fragmented.
13. A method according to any preceding claim, wherein the cleavage agent employed comprises an endopeptidase or a chemical cleavage agent.

14. A method according to claim 13, wherein the cleavage agent employed comprises Lys-C endopeptidase, a thiocyanate compound, cyanogen bromide, BNPS-skatole, trypsin, chymotrypsin, and/or thrombin.

15. A method according to any of claims 3-14, wherein the capping agent comprises one or more of an iodoacetate compound, an isocyanate compound, a silyl compound, an anhydride, a vinylsulphone compound and a vinyl pyridine derivative.

16. A method for determining the expression of one or more proteins in a tissue, which method comprises characterising a population of polypeptides according to a method as defined in any preceding claim.

17. A method for assaying for one or more specific polypeptides in a sample, which method comprises characterising a population of polypeptides according to a method as defined in any of claims 1-15, and determining the presence or absence of the one or more specific polypeptides from the presence or absence of one or more specific fragments corresponding to the polypeptides.

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FOR UTILITY/DESIGN  
CIP/PCT NATIONAL/PLANT  
ORIGINAL/SUBSTITUTE/SUPPLEMENTAL  
DECLARATIONS

RULE 63 (37 C.F.R. 1.63)  
DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION  
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PW  
FORM

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the INVENTION ENTITLED CHARACTERISING  
POLYPEPTIDES THROUGH CLEAVAGE AND MASS SPECTROMETRY

the specification of which (CHECK applicable BOX(ES))

X A. ☐ is attached hereto.

BOX(ES) → B. ☐ was filed on \_\_\_\_\_ as U.S. Application No. \_\_\_\_\_

→ C. ☒ was filed as PCT International Application No. PCT/ GB99/03258 on October 1, 1999

and (if applicable to U.S. or PCT application) was amended on \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. Except as noted below, I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application

PRIOR FOREIGN APPLICATION(S)

Number	Country	Day/MONTH/Year Filed
9821393.7	Great Britain	1 October 1998

Date first Laid-  
open or Published

Date Patented  
or Granted

Priority NOT Claimed

If more prior foreign applications, X box at bottom and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U.S.C. 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S)

Application No. (series code/serial no.)	Day/MONTH/Year Filed
PCT/GB99/03258	1 October 1999

Status  
pending, abandoned, patented  
pending

Priority NOT Claimed

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Winthrop LLP, Intellectual Property Group, 1100 New York Avenue, N.W., Ninth Floor, East Tower, Washington, D.C. 20005-3918, telephone number (202) 861-3000 (to whom all communications are to be directed), and the below-named persons (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete names/numbers below of persons no longer with their firm and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above firm and/or a below attorney in writing to the contrary.

Name	First	Middle Initial	Family Name	31204	Anthony L. Miele	34393	
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G. Lloyd Knight	17698	G. Paul Edgell	24238	Michael R. Dzwonczyk	36787	Robert J. Walters	40862
Kevin E. Joyce	20508	Lynn E. Eccleston	35861	Jack S. Barufka	37087	Brian J. Beatus	38825
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(1) INVENTOR'S SIGNATURE:

Date: 30/5/01

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(2) INVENTOR'S SIGNATURE:

Date: 22/5/01

2-00	Andrew	Hugin	Thompson
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"X" box ☐ FOR ADDITIONAL INVENTORS, and proceed on the attached page to list each additional inventor.

☐ See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. P0279469

(M#)



**Rule 56(a) & (b) = 37 C.F.R. 1.56(a) & (b)**  
**PATENT AND TRADEMARK CASES - RULES OF PRACTICE**  
**DUTY OF DISCLOSURE**

- (a) ... Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the [Patent and Trademark] Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability... (b) information is material to patentability when it is not cumulative and (1) It also establishes by itself, or in combination with other information, a prima facie case of unpatentability of a claim or (2) refutes, or is inconsistent with, a position the applicant takes in: (i) Opposing an argument of unpatentability relied on by the Office, or (ii) Asserting an argument of patentability

**PATENT LAWS 35 U.S.C.**

**§102. Conditions for patentability; novelty and loss of right to patent**

A person shall be entitled to a patent unless--

- (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent or
- (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States, or
- (c) he has abandoned the invention, or
- (d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months\* before the filing of the application in the United States, or
- (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or
- (f) he did not himself invent the subject matter sought to be patented, or
- (g) before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other.

**§103. Condition for patentability; non-obvious subject matter**

- (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made. . . .
- (c) Subject matter developed by another person, which qualified as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

\* Six months for Design Applications (35 U.S.C. 172).